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Functional Differences between Heme Permeases: *Serratia marcescens* HemTUV Permease Exhibits a Narrower Substrate Specificity (Restricted to Heme) Than the *Escherichia coli* DppABCDF Peptide-Heme Permease[▽]

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Serratia marcescens hemTUV **genes encoding a potential heme permease were cloned in** ***Escherichia coli*** **recombinant mutant FB827** ***dppF*****::Km(pAM 238-hasR). This strain, which expresses HasR, a foreign heme outer membrane receptor, is potentially capable of using heme as an iron source. However, this process is invalidated due to a** ***dppF*****::Km mutation which inactivates the Dpp heme/peptide permease responsible for heme, dipeptide, and δ-aminolevulinic (ALA) transport through the** ***E. coli*** **inner membrane. We show here that** ***hemTUV*** **genes complement the Dpp permease for heme utilization as an iron source and thus are functional in** ***E. coli*****. However,** ***hemTUV*** **genes do not complement the Dpp permease for ALA uptake, indicating that the HemTUV permease does not transport ALA. Peptides do not inhibit heme uptake in vivo, indicating that, unlike Dpp permease, HemTUV permease does not transport peptides. HemT, the periplasmic binding protein, binds heme. Heme binding is saturable and not inhibited by peptides that inhibit heme uptake by the Dpp system. Thus, the** ***S. marcescens*** **HemTUV permease and, most likely, HemTUV orthologs present in many gram-negative pathogens form a class of heme-specific permeases different from the Dpp peptide/heme permease characterized in** ***E. coli*****.**

Heme, the prosthetic group of various hemoproteins, is also a major iron source for microbes. To retrieve iron, bacteria import heme as a whole molecule and degrade it in the cytoplasm. In gram-negative bacteria, heme is actively transported through the outer membrane by specific proton motive force-powered receptors and through the inner membrane by periplasmic binding protein-dependent ABC permeases. Most outer membrane heme receptors directly extract free or host hemoprotein-bound heme from the medium. Such receptors are widespread among gram-negative species. The best-characterized receptors are *Yersinia enterocolitica* HemR (20) and *Shigella dysenteriae* ShuA (28). A more elaborate heme uptake system is present in fewer species, such as *Serratia marcescens* and *Pseudomonas* and *Yersinia* species. This system involves bacterial extracellular proteins called hemophores (10). Because of their high affinity for heme, hemophores extract this compound from various host hemoproteins and convey it to hemophore-specific outer membrane receptors (HasR) that internalize only heme (27). HasR proteins form a conserved subfamily of heme receptors which exhibit low levels of sequence similarity with other heme receptors. Nevertheless, the *S. marcescens* HasR receptor allows free and hemoglobin-bound heme uptake through the outer membrane (4).

In many gram-negative pathogens, heme permeases have similar sequences and organizations and are comprised of one periplasmic heme binding protein, HemT, one transmembrane protein, HemU, and one ABC protein, HemV (21). Heme permease structural genes are usually clustered, and only one copy is present in each bacterial genome. The heme permease gene set is localized in an iron-regulated operon that often also includes a gene encoding one heme outer membrane receptor (26).

Pathogenic *Escherichia coli* strains have genes that are homologous to *hemTUV* and are linked to the structural gene for the heme outer membrane receptor. This cluster has a similar genetic organization and chromosome localization in many *E. coli* strains, suggesting that it was acquired by horizontal transfer (28).

On the other hand, *E. coli* K-12 lacks a heme outer membrane receptor and heme permease orthologs and is unable to use exogenously added heme as an iron source. Nevertheless, a recombinant clone expressing only a foreign heme outer membrane receptor, such as ShuA, HemR, or HasR, can use heme as an iron source, suggesting that there is an unidentified heme permease. In previous work, we showed that *E. coli* heme permease consists of the dipeptide ABC transporter DppBCDF functioning with one of two optional periplasmic binding proteins, either MppA, the L-alanyl-γ-D-glutamyl-*meso*-diaminopimelate binding protein (14), or DppA, the dipeptide binding protein (1). Purified DppA and MppA proteins are able to bind heme in vitro with binding constants of approximately 10 and 50 μM, respectively. Peptides compete for heme binding to DppA and MppA in vitro, while in vivo they inhibit heme transport (9). The DppA/MppA DppBCDF

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permease also transports δ-aminolevulinic acid (ALA), a heme precursor that resembles the GG dipeptide (25).

Two other periplasmic DppA homologs, the *E. coli* NikA protein and *Haemophilus influenzae* HbpA, also bind heme (18) (5). While periplasmic binding proteins are usually specific for one substrate or closely related structural homologs, it is surprising that NikA, DppA, and MppA bind heme in addition to their cognate substrates. However, it is not known whether HbpA and NikA also bind peptides in addition to heme.

The present work was undertaken to determine whether other heme binding periplasmic proteins can bind peptides. The *hemTUV* genes of *S. marcescens* were cloned and expressed in an *E. coli* recombinant strain having a heme outer membrane receptor enabling heme uptake through the outer membrane and carrying a *dppF*::Km mutation which inactivated the Dpp-heme permease.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* strains FB8 (wild type, F^-) and FB827 (*entF*::Tn*10*) have been described previously (16). POP3 (*araD139* Δ*lacU169 rpsL relA thi*) and POP3 *hemA* were obtained from a laboratory collection. FB827 *dppF*::Tn*5* has been described previously (9). *S. marcescens* DB11 was described by Kurz et al. (7) and was a gift from J. Ewbank. Mutations were introduced into strains by phage P1 transduction.

pAM 238 and pTRC99 were obtained from a laboratory collection, and pAM 238-hasR has been described previously (8). pTRC99-hemTUV and pTRC99-hemT are described in this paper.

Media and growth conditions. Hemin, bovine hemoglobin, ALA, 2,2′dipyridyl (Dip), and isopropyl-β-D-thiogalactopyranoside (IPTG) were obtained from Sigma Chemical Company (Lyon, France). The hemoglobin concentration was calculated on the basis of the heme monomer. Hemoglobin and ALA solutions were sterilized by passage through 0.45-μm-pore-size filters. Hemin was dissolved immediately before use in a minimal volume of 0.1 N NaOH, filtered, and diluted with the appropriate buffer to obtain the desired concentration. Bacteria were grown aerobically at 37°C in LB rich medium, in M63, or in M63 without added iron salt (M63*). All minimal media were supplemented with 0.4% glucose. When required, Dip was added at a final concentration of 60 μM to M63*. IPTG (1 mM) was added to induce the pTRC99 *trc* promoter. Antibiotics were added at the following final concentrations: ampicillin, 50 μg ml^{-1}; kanamycin, 25 μg ml^{-1}; spectinomycin, 50 μg ml^{-1}; tetracycline, 10 μg ml^{-1}; and chloramphenicol, 15 μg ml^{-1}. For each strain, the carbon source and the appropriate antibiotics were added to solid and liquid media (data not shown). All cultures were grown with aeration at 37°C, and the optical density at 600 nm (OD_{600}) was determined.

Growth promotion assays. Cultures of strain FB827(pAM 238-hasR) carrying various mutations and plasmids were grown in M63 containing glucose to an OD_{600} of 1, and 100-μl aliquots were mixed with 3.5 ml of M63* soft agar (0.7% agar) and poured onto M63* plates containing Dip supplemented with 1 mM IPTG to induce the pTRC99-encoded genes. Aliquots (50 μl) of bovine hemoglobin at various concentrations were placed in wells punched in the solidified agar. The plates were incubated overnight at 37°C, and the radius of the growth halo around each well was measured. All experiments were repeated three times.

To test its ability to use ALA, heme auxotroph strain POP3 *hemA* carrying various mutations and plasmids was streaked on M63 plates containing the appropriate antibiotics and supplemented with either 0.5 or 5 μg/ml of ALA. Colony sizes were measured after 48 h of incubation at 37°C.

Genetic techniques. Preparation of P1 lysates and transduction were performed as described by Miller (11). Competent cells were prepared by the calcium chloride method.

Extraction and manipulation of plasmids. Standard methods were used for isolation of plasmid DNA, cloning, restriction enzyme analysis, and transformation.

Plasmid construction. Plasmids encoding HemTUV and HemT were constructed by amplification of *S. marcescens* DB11 genomic DNA using complementary oligonucleotides (sequences available upon request). Amplified fragments with appropriate restriction nuclease recognition sites were inserted into pTRC99. Amplified gene sequences were checked by DNA sequencing.

Production and purification of HemT protein. HemT was purified by using the procedure used to purify DppA and MppA described previously (9). Briefly, 6 liters of a POP3(pTRC99-HemT) cell culture was grown at 37°C in M63. When the culture reached an OD_{600} of 0.2, IPTG was added to a final concentration of 1 mM. The culture was grown for an additional 4 h at 37°C to an OD_{600} of 0.8 and harvested by centrifugation for 15 min at 8,000 × *g* at 4°C. The cell pellet was washed once in TE buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA), resuspended at room temperature in 480 ml of 30% (wt/vol) sucrose (OD_{600}, 10), and incubated for 30 min at room temperature. Then EDTA (pH 8) was added to a final concentration of 10 mM, and cells were incubated again for 15 min at room temperature. The cell suspension was centrifuged at 10,000 × *g* for 30 min at 4°C. The supernatant containing the periplasmic shock fluid was concentrated by 80% ammonium sulfate precipitation and then extensively dialyzed against 50 mM Tris-HCl (pH 7.5)–80 mM NaCl at 4°C.

Samples of concentrated osmotic shock fluid were first purified by cation-exchange chromatography performed as described previously for DppA (9). This was followed by anion-exchange chromatography using gels preequilibrated with 20 mM Tris-HCl (pH 8.5). Elution was performed with 20 mM Tris-HCl (pH 8.5)–1 M NaCl. Fractions were collected, and their HemT contents and purity were evaluated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Fractions containing pure HemT that produced a single protein band at an apparent molecular mass of 27 kDa were pooled and concentrated by using an Amicon Ultra 15 with a 10,000-molecular-weight cutoff (Millipore). The N-terminal amino acid sequence was determined by the Plateforme d'Analyze et de Microséquence des Protéines of the Institut Pasteur.

Nondenaturing PAGE and detection of heme by chemiluminescence. Aliquots (30 μl) of purified HemT (concentration, 3×10^{-6} M) were incubated at room temperature for 30 min with heme at various concentrations, including 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} M. Heme-loaded samples were separated by PAGE (at 4°C in the absence of SDS), and the proteins were transferred to nitrocellulose filters. Heme complexed with protein bands on the gel retained its intrinsic peroxidase activity, which was detected by chemiluminescence (ECL Plus; Amersham) as described previously (24). The signal was measured either by autoradiography on film or with a Storm Imager.

Peptide inhibition of heme binding to HemT was tested by adding to 30-μl purified HemT aliquots (concentration, 3×10^{-6} M) either heme alone (10^{-4} M) or a mixture of heme (10^{-4} M) and peptides Ala-Ala (AA) and Pro-Phe-Lys (PFK) (10^{-4} M each). Peptone and tryptone inhibition of heme binding to HemT was also tested by adding to 10-μl purified HemT aliquots (concentration, 3×10^{-6} M) a mixture of heme (10^{-4} M), peptone (final concentration, 7%), and tryptone (final concentration, 7%). The mixtures were separated by PAGE, and heme was detected as described above.

RESULTS

***S. marcescens* DB11 *hemTUV* genes are functional in *E. coli* for iron heme utilization.** Using the *S. marcescens* DB11 genomic sequence database (http://www.sanger.ac.uk/Projects/S_marcescens/), we identified only one set of *hemTUV* orthologs. The *S. marcescens* HemT protein shared 72, 37, and 33% sequence identity with periplasmic heme binding HemT proteins of *Y. enterocolitica*, *S. dysenteriae*, and *Pseudomonas aeruginosa*, respectively. The *S. marcescens* inner membrane component HemU and the ABC protein HemV belong to the well-conserved family of siderophore, heme, and vitamin B_{12} ABC transporters. The *hemTUV* genes are contiguous, with one gene located next to another, and are located in a potentially iron-regulated operon that also contains the *hemR* gene encoding a potential heme outer membrane receptor. This genetic organization is generally found for most *hemTUV* genes.

The DB11 *hemTUV* genes were amplified by PCR and cloned into pTRC99. Strain FB827(pAM 238-hasR) and the isogenic mutant FB827 *dppF*::Km(pAM 238-hasR) were transformed with pTRC99-dppABCDF or pTRC99-hemTUV. Strains were grown in M63 to an OD_{600} of 1 and tested for growth on M63* plates containing Dip and 1 mM IPTG. Be-

TABLE 1. Complementation of *dppF*::Km mutation for iron heme utilization by *hemTUV* and *hemT* genes[a]

Strain	Growth on M63* containing Dip around wells containing hemoglobin at a concn of:			
	50 μM	10 μM	5 μM	1 μM
FB827(pAM 238)	−	−	−	−
FB827(pAM 238-hasR)	+++	++	+	−
FB827 *dppF*::Km(pAM 238-hasR)	−	−	−	−
FB827 *dppF*::Km(pAM 238-hasR)(pTRC99-dppABCDF)	+++	++	+	−
FB827 *dppF*::Km(pAM 238-hasR)(pTRC99-hemTUV)	++	−	−	−
FB827 *dppA*::Km *mppA*::Cm(pAM 238-hasR)	−	−		
FB827 *dppA*::Km *mppA*::Cm(pAM 238-hasR)(pTRC99-hemT)	−	−		
FB827(pAM 238-hasR)(pTRC99-hemT)	−	−		

[a] Aliquots (50 μl) of bovine hemoglobin at the concentrations indicated were placed in wells punched into solidified agar. The plates were incubated overnight at 37°C, and the radius of the growth around each well was measured. +++, radius of 10 mm; ++, radius of 6 mm; +, radius of 2 mm; −, no growth around the wells. All experiments were repeated three times.

cause of iron restriction, none of the tested strains could grow on these plates. Wells were punched into solidified agar and filled with 50 μl of bovine hemoglobin at concentrations ranging from 1 to 50 μM. As shown previously by another growth promotion assay (9), FB827(pAM 238-hasR) could grow only around hemoglobin-containing wells; the lowest hemoglobin concentration that resulted in a significant halo was 5 μM, and neither strain FB827(pAM 238) lacking heme outer membrane receptor HasR nor mutant FB827 *dppF*::Km(pAM 238-hasR) could grow on these plates around the wells containing hemoglobin at concentrations up to 50 μM (hemoglobin concentrations equal to or higher than 100 μM could not be used as they might promote nonspecific growth in this test). FB827 *dppF*::Km(pAM 238-hasR)(pTRC99-dppABCDF) grew as well as parental strain FB827(pAM 238-hasR) around the hemoglobin-filled wells (Table 1).

FB827 *dppF*::Km(pAM 238-hasR)(pTRC99-hemTUV) grew around the hemoglobin-containing wells, albeit less efficiently. The lowest hemoglobin concentration allowing significant growth was 50 μM (Table 1). Thus, the *S. marcescens hemTUV* genes complement FB827 *dppF*::Km(pAM 238-hasR) for iron heme utilization, indicating that the HemTUV permease is functional in *E. coli* and could replace the Dpp permease for utilization of heme as an iron source (Table 1). The slightly lower efficiency of the HemTUV permease than of the Dpp permease might have been caused by various factors, such as lower expression or stability in a heterologous host.

Peptides do not inhibit iron heme utilization via HemTUV permease. The dipeptide AA has a strong affinity for DppA (19), and the tripeptide PFK is transported by MppA-OppBCDF permease (14). In previous work we showed that a combination of these two peptides inhibits heme utilization via the Dpp permease (9). We thus tested whether such competition also occurs via the HemTUV permease. Strains FB827 *dppF*::Km(pAM 238-hasR)(pTRC99-dppABCDF) and FB827 *dppF*::Km(pAM238-hasR)(pTRC99-hemTUV) were grown and tested as described above on M63* plates containing Dip and 1 mM IPTG with wells filled with either 50 μM hemoglobin alone or a mixture of hemoglobin and various peptides, as indicated in Table 2. Growth of FB827 *dppF*::Km(pAM 238-hasR)(pTRC99-dppABCDF), which has a functional Dpp permease, was inhibited around wells containing a mixture of hemoglobin and either peptone (20%) or tryptone (20%) or containing a combination of the dipeptide AA and the tripeptide PFK (100 μM each). However, the growth of FB827 *dppF*::Km(pAM 238-hasR)(pTRC99-hemTUV) was the same in all wells independent of the presence of peptides, indicating that none of the peptides inhibited iron heme utilization via HemTUV (Table 2).

TABLE 2. Comparison of various peptide effects on iron-heme utilization via DppA/MppA DppBCDF and HemTUV permeases[a]

Strain	Growth on M63* containing Dip around wells containing 50 μM hemoglobin and various peptides			
	No addition	AA + PFK	Peptone	Tryptone
FB827(pAM 238-hasR)	+++	−	−	−
FB827 *dppF*::Km(pAM 238-hasR)	−	−	−	−
FB827 *dppF*::Km(pAM 238-hasR)(pTRC99-dppABCDF)	+++	−	−	−
FB827 *dppF*::Km(pAM 238-hasR)(pTRC99-hemTUV)	++	++	++	++

[a] Aliquots (50 μl) of 50 μM bovine hemoglobin alone, with a mixture of peptides AA and PFK each at a concentration of 100 μM, with 20% peptone, or with 20% tryptone were placed in wells punched into solidified agar. The plates were incubated overnight at 37°C, and growth was determined as described in Table 1, footnote *a*.

ALA is not transported by the HemTUV permease. Dpp permease is required for ALA uptake at ALA concentrations equal to or lower than 0.5 μg/ml. At ALA concentrations higher than 0.5 μg/ml, other permeases contribute to ALA uptake (9). Heme auxotrophic mutants, defective in ALA synthesis, were tested for fast aerobic growth on M63 plates in the presence of increasing ALA concentrations. Whereas POP3 *hemA* grew well on M63 plates containing 1 mM IPTG supplemented with 0.5 μg/ml of ALA, neither strain POP3 *hemA dppF*::Km nor strain POP3 *hemA dppF*::Km(pTRC99-hemTUV) could grow at this ALA concentration (Table 3). All

TABLE 3. Complementation of the *dppF*::Km mutation for ALA utilization by *hemTUV* and *hemT* genes[a]

Strain	Growth on M63 containing:	
	0.5 μg/ml ALA	5 μg/ml ALA
POP3 *hemA*	+++	+++
POP3 *hemA dppF*::*Km*	−	+++
POP3 *hemA dppF*::*Km*(pTRC99-hemTUV)	−	+++
POP3 *hemA*(pTRC99-hemT)	+++	+++

[a] Strain POP3 *hemA* or POP3 *hemA dppF*::*Km* carrying the *hemTUV* or *hemT* gene on pTRC99 was streaked on M63 containing glucose supplemented with 0.5 or 5 μg/ml ALA and incubated for 48 h at 37°C. +++, normal-size colonies like those of the POP3 *hemA*$^+$ strain; −, no growth after 48 h. All experiments were repeated three times.

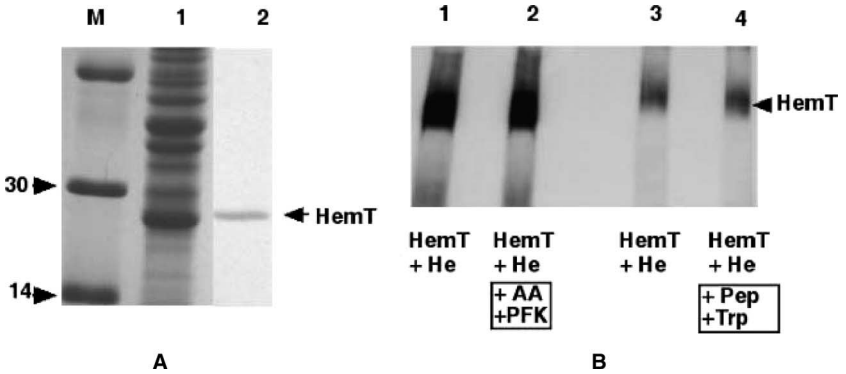

FIG. 1. Purification and heme binding properties of HemT. (A) SDS-PAGE analysis of periplasmic and purified HemT. Lane M, molecular mass markers (molecular masses [in kDa] are indicated on the left); lane 1, concentrated osmotic shock fluid of POP3(pTRC99-hemT); lane 2, purified HemT protein. (B) Enhanced chemiluminescence detection after nondenaturing PAGE and transfer onto a nitrocellulose membrane. Proteins were incubated at room temperature for 30 min either with heme or with a mixture of heme and peptides or only buffer as indicated below the lanes. The final concentration of heme was 100 μM; the final concentration of the AA and PFK peptides was 100 μM each. Peptone and tryptone were each added at a final concentration of 20% (+Pep+Trp). Mixtures were separated by PAGE (at 4°C in the absence of SDS), and the proteins were transferred to nitrocellulose filters. Heme complexed with protein bands on the gel retained intrinsic peroxidase activity, which was detected by chemiluminescence (ECL Plus; Amersham). The signal was measured either by autoradiography on film or with a Storm Imager.

strains formed normal-size colonies at higher ALA concentrations. Thus, unlike the DppA/MppA DppBCDF permease, the HemTUV permease is not involved in high-affinity ALA uptake.

HemT overexpression inhibits iron heme utilization. The *hemT* gene was amplified by PCR and cloned into pTRC99. To test the activity of the HemT protein, strains FB827(pAM 238-hasR) and FB827 *dppA*::Km *mppA*::Cm(pAM 238-hasR) were transformed with pTRC99-hemT and tested for iron heme acquisition as described above. The growth of transforming cells was tested as described above on M63* plates containing Dip and 1 mM IPTG with wells filled with 50 μM hemoglobin. FB827 *dppA*::Km *mppA*::Cm(pAM 238-hasR) (pTRC99-hemT) did not grow around the wells, indicating that HemT does not complement *dppA* or *mppA* mutations. Moreover, FB827(pAM 238-hasR)(pTRC99-hemT) also did not grow around the wells, indicating that HemT was either sequestering heme in the periplasm or interacting with the Dpp permease in such a way that it blocked heme uptake (Table 1).

HemT overproduction does not inhibit ALA transport. To determine whether HemT sequesters heme or inhibits the Dpp permease, we tested the effect of HemT overproduction on the ability of organisms to use ALA at low concentrations. POP3 *hemA*(pTRC99-hemT) grew as well as POP3 *hemA* on M63 plates containing glucose and 1 mM IPTG supplemented with 0.5 μg/ml of ALA (Table 3). Thus, HemT does not bind ALA and does not inhibit Dpp permease. It might withhold heme from Dpp permease.

HemT "in vitro" properties. POP3(pTRC99-hemT) was grown in M63 with 1 mM IPTG to induce the *hemT* gene and was osmotically shocked to release its periplasmic contents. A protein band induced by IPTG and with an apparent molecular mass of 27 kDa was detected in the shock fluid (Fig. 1A, lane 1). The N-terminal sequence of the HemT protein was AQR IVS, indicating that the *hemT* gene encodes a 23-residue, cleavable, N-terminal signal peptide. This signal peptide had a twin-arginine motif and an overall sequence similar to a Tat signal sequence (17). We thus tested whether HemT is translocated to the periplasm by the Tat system, which delivers folded proteins. The HemT amounts in the periplasm were similar in *tat*$^+$ and *tat* backgrounds, indicating that HemT translocation is Tat independent (data not shown). HemT was purified as described in Materials and Methods. This resulted in a single band on SDS-PAGE gels at an apparent molecular mass of 27 kDa (Fig. 1 A, lane 2). Pure HemT was incubated with 10^{-4} M heme and electrophoresed on a nondenaturing PAGE gel, which allowed separation of free heme, apoprotein, and heme-loaded protein without dissociation of heme from the protein. Heme bound to the protein was detected by chemiluminescence (Fig. 1 B, lanes 1 and 3). HemT was incubated with various concentrations of heme. The samples were analyzed as described above, and the heme bound to proteins was determined by enhanced chemoluminescence. Heme binding was saturable with a dissociation constant for heme less than 10^{-6} M (data not shown).

Peptides do not compete for heme binding. To test whether peptides inhibit heme binding to HemT, 30 μl of HemT (3×10^{-6} M) was incubated either with heme alone (Fig. 1B, lane 1) or in the presence of a mixture of the dipeptide AA and the tripeptide PFK (each at a concentration of 10^{-4} M). There was no inhibition of heme binding by these peptides (Fig. 1B, lane 2). To test the effect of other oligopeptides on heme binding to HemT, 10 μl of HemT (3×10^{-6} M) was incubated either with heme alone (Fig. 1B, lane 3) or with a mixture of peptone and tryptone (7% each) (Fig. 1B, lane 4). There was no inhibition of heme binding by the peptone-tryptone mixture. Thus, peptides do not inhibit heme utilization "in vivo" or heme binding to HemT "in vitro."

DISCUSSION

Our data indicate that the *S. marcescens* HemTUV permease is functional in *E. coli* and replaces the Dpp permease for heme uptake through the inner membrane when HasR is present to promote heme transport through the outer membrane. The two permeases, however, have different properties.

Whereas the Dpp permease transports di- and tripeptides, ALA, and heme, the HemTUV permease transports only heme. Heme binding to purified periplasmic HemT protein is not inhibited by peptides. In most cases periplasmic binding proteins are responsible for substrate recognition and specific transfer to their ABC dedicated transporters. Thus, it appears that periplasmic heme binding protein constituents of ABC permeases belong to two classes. One class is comprised of large binding proteins, such as DppA, MppA, NikA, and HbpA, which recognize substrates other than heme. DppA and MppA are involved in heme delivery to the Dpp transporter in *E. coli*. HbpA is also involved in heme uptake in *H. influenzae*, but the corresponding heme ABC transporter has not been identified yet. Heme-loaded NikA is not involved in heme uptake in *E. coli*, but it might be involved in cytochrome maturation (18). This class is related to the Opp-like transporters, which are known to be involved in the transport of a large variety of substrates, such as sugar in archaea and thermophilic bacteria (12), proline betaine in *Rhizobiaceae* (2), and agrocinopine in *Agrobacterium tumefaciens* (6). The second class of periplasmic heme binding proteins includes smaller proteins, such as *S. marcescens* HemT, *S. dysenteriae* ShuT (3), and *P. aeruginosa* PhuT (23). We show here that HemT is specific for heme. It is likely that this is also the case for the other heme binding proteins belonging to this class.

The contribution of each type of permease to heme acquisition by bacterial pathogens is actually not known. BLAST searches for putative heme permeases did not reveal any *hemTUV* orthologs in several species, such as *Neisseria* and *Haemophilus* species, which have functional heme outer membrane receptors and which are able to use heme as an iron source (15). It is tempting to speculate that in these organisms heme is taken up by peptide permeases. In addition, *hemTUV* gene inactivation in various mutants, such as *Y. enterocolitica*, *Yersinia pestis*, and *Vibrio cholerae* mutants, does not abolish heme uptake, suggesting that in these mutants peptide permeases may take over from the HemTUV permeases (13, 20, 22). Genetic inactivation of each permease and of both types of permeases in these strains should help elucidate the relative role of each type of permease in heme uptake.

A priori, heme transport through the inner membrane by *E. coli* K-12, an organism lacking an outer membrane heme receptor, seems meaningless. However, it is likely that the peptide/heme permease is involved in endogenous heme recycling.

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